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THE EFFECT OF SOME MILK SALTS ON THE STABILITY OF THE CALCIUM CASEINATE-PHOSPHATE COMPLEX

by

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ABSTRACT

The stability of the calcium caseinate-phosphate complex of milk to ethanol and rennet, and its relationship to various salts in milk was examined. A total of 107 milk samples from Holstein cows showed a wide variation in stability. Coagulation was caused by ethanol solutions ranging in strength from 66 - 88%, with an average of 69.3%. The clotting time which was measured by an automatic clot-timer varied from 66 - 130 seconds for 107 milk samples, with an average of 87.9 seconds.

The milk samples were analyzed for total and soluble calcium, magnesium, citrate and inorganic phosphate, and ionic calcium concentrations. No close or consistant relationship was observed between the stability as measured by ethanol and rennet clotting time, and the chemical composition of the samples. However, the various variables determined showed a low to moderate degree of correlation to the stability.

In general, there appeared to be a moderate degree of correlation between the clotting times and alcohol stability of the samples. Some of the milk constituents determined were found to be interrelated to one another. There was a moderate degree of correlation between the total calcium and soluble calcium and a greater degree of correlation between the soluble calcium and ionic calcium contents of the milk samples.

The salt balance theory was found inadequate in explaining the variation in the alcohol stability of the milk samples.

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THE EFFECT OF SOME MILK SALTS ON THE STABILITY OF THE CALCIUM CASEINATE-PHOSPHATE COMPLEX

INTRODUCTION

The stability of milk protein may be defined as the ability of the casein to remain in colloidal suspension and of lactalbumin and lactoglobulin to remain in solution when milk is subjected to heat or other modifying influences. A study of many aspects of protein stability, in addition to being of purely chemical interest is of practical importance. The successful manufacture of evaporated milk, sweetened condensed milk and cheese for example is largely dependent on the behaviour of the milk proteins, especially casein.

Although empirical methods have been devised to overcome some of the difficulties in the processing of milk, such as protein coagulation during the sterilization of evaporated milk, the age thickening of condensed milk and slow or incomplete coagulation with rennet, the underlying causes of these defects are far from being completely understood. That this should be so despite intensive research is not surprising in view of the variability in the composition of milk and the complex nature of some of its constituents.

It has been suggested from time to time that the concentration of certain milk salts is of prime importance in determining the stability of the caseinate complex, but the results are by no means conclusive. This may have resulted from the inadequacy of existing analytical methods or from the fact that in any one investigation only a small number of the many compositional variables in milk, which might conceivably effect stability, has been determined. Another possible reason is that each milk sample is a unique colloidal system and that therefore no general relation between composition and stability can be arrived at. However, there is no justification as yet for resorting to the latter hypothesis. There is need for an investigation in which milks of widely differing stability to several coagulating agents are analysed in detail and an attempt made to relate stability to chemical composition. This was the object of the present investigation. The coagulating agents used were ethanol and rennet, since these are of considerable practical importance.

General Principles Underlying Colloid Stability and Coagulation

Fundamentally the stability of colloidal dispersions depends upon diffusion resulting from thermal agitation of the dispersed micelles (Brownian motion) and the molecules of the continuous phase. If, however, colloidal micelle stick together, 'aggregates' build up and coagulation rather than diffusion takes place.

In a colloidal dispersion of proteins, both electric charge and hydration effect the stability. With many proteins hydration predominates to such an extent that the dispersions act chiefly as emulsoid sols, but with others charge and hydration are more nearly equal in importance, and dispersions exhibit properties intermediate between those of supensoids and emulsoids. With all proteins the electric charge varies in sign and magnitude in response to alterations in the acidity and alkalinity of the medium. There is an intermediate pH where the number of positive charges and negative charges on the protein are equal. This is the isoelectric point for that protein. At the isoelectric point many properties of the protein solution are at either a maximum or a minimum. Among the latter are the viscosity, osmotic effects, such as osmotic pressure, electrical conductivity, diffusion, electrokinetic movement, solubility and swelling; while the coagulability by heat or by alcohol is at a maximum. Coagulation of sols is effected by sufficiently decreasing the electric charge or hydration, whichever is the predominant factor in stability, or by decreasing both charge and hydration.

Colloidal Stability and Coagulation of Milk

The several constitutents of the disperse phase of milk differ greatly in colloid characteristics. The calcium phosphate is entirely suspensoid in nature, and in milk serum would be unstable if the proteins were not present. It must therefore be regarded

as a suspensoid protected by one or more of the proteins. It is usually assumed that the protection is rendered by the calcium caseinate. Calcium caseinate exhibits properties intermediate between those of suspensoids and emulsoids. It is suspensoid in that it coagulates at or near its isoelectric point, it is moderately sensitive to coagulation by inorganic salts, and the coagulating effectiveness of salts is governed more by the valence of their ions than by position in the lyotropic series. On the other hand, its protective power, ease of dispersion in acids and alkalies and formation of jellies give it marked emulsoid characteristics. Lactalbumin and lactoglobulin are predominately emulsoid in character, they remain dispersed at their isoelectric points and are coagulated by inorganic salts only at high concentrations, the effectiveness of salts depending chiefly upon their position in the lyotropic series.

The exact role of lactalbumin and lactoglobulin in the coagulation of milk is not known, but apart from colostrum whose content of these heat sensitive proteins is very high, the stability of the major protein casein is thought to determine the coagulability of milk. The calcium caseinate in milk or more precisely the mixture of α-, β- and γ-caseinates is combined or associated with varying amounts of inorganic material thought to be mainly tricalcium phosphate. The calcium caseinate-calcium phosphate mixture or complex is colloidally dispersed as globular micelles ranging in diameter from a few millimicrons to 800 millimicrons,

with an average diameter of about 100 millimicrons. It is generally accepted that the protein dispersion remains stable by virtue of the hydration and negative electrical charges, i.e. the electrokinetic potential of the micelles. A progressive decrease in either causes micelles to aggregate and eventually to appear as clots of coagulated proteins. It seems probable that one or both of these changes must precede coagulation whatever the coagulating agent.

The stability of the caseinate complex in milk will therefore depend not only on the initial physical or chemical composition of the milk, but, in some instances, also on the rate or extent of secondary reactions induced by the coagulating agent.

REVIEW OF THE LITERATURE

Milk Salts

According to Jenness and Patton (34), the salts of the milk include those constituents except hydrogen ions and hydroxyl ions that are present as ions or in equilibrium with ions. The salts of milk in the usual sense of the expression comprise a small and relatively simply constituted fraction of the milk solids made up mainly of chlorides, phosphates, citrates and bicarbonates of sodium, potassium, calcium and magnesium. In a more extended sense the milk salts include also the proteins, since these also carry positively and negatively charged groups and can form salts with ions. According to Pyne (49), the milk salts have a marked influence on the condition and stability of the milk proteins, casein in particular, and relatively insignificant variations in the salt composition can often exercise quite disproportionate effects in this regard.

Partition Between Dissolved and Colloidal Phases

Certain of the milk salts, e.g. the chlorides, and the compounds of sodium and potassium, are sufficiently soluble to be present entirely or almost entirely in dissolved form, that is in the milk serum. Others, in particular calcium phosphate, are much less soluble, and being present in amounts which exceed their

solubilities at the normal pH of milk, exist partly in dissolved and partly in insoluble or rather colloidal form and, in fact, in close association with casein. As the influence of this type of salt on the properties of milk differs considerably according to the phase in which it is situated, a correct assessment of salt partition between serum and milk colloids is of primary importance.

Various methods of study have been used to separate the dissolved from the colloidal phases in milk in order to study the partition of salts between them. Of course, the distinction between the states of solution and colloidal dispersion results from arbitrarily drawing the line at some point of particle size defined by the operations used for separation. For the milk salts, however, a fairly sharp separation is not unduly difficult chiefly because the insoluble or colloidal salts occur mainly in association with the relatively coarse colloidal micelles of casein. A variety of methods is available and in common use for this separation; these include dialysis, ultrafiltration, high speed centrifugation, and the use of casein coagulating enzymes such as rennin. All of these methods have been used almost indiscriminately for the purpose both in earlier and more recent work.

Earlier determinations of White and Davies (71) made on ultrafiltrates from herd milk samples indicate, in general agreement with the previous findings, that about 31% of the calcium, 65% of the magnesium, 53% of the inorganic phosphorus and 89% of the citrate of milk are present in the serum. The corresponding

average values reported by Verma and Sommer (66) from analysis of rennet whey are 39% for calcium, 73% for magnesium and 90% for citrate. These authors' values for phosphorus are not given here as they refer to total, not inorganic phosphorus. The more recent results of Davies and White (17), based on analysis of milk diffusate prepared at 20°C and corrected for bound water, differ little from their earlier ones, except that soluble citrate is now assessed at 94% instead of the earlier 89%. They also observed that small amounts of calcium and citrate are retained by the ultrafiltration membrane due to the 'sieving effect'. This phenomenon is described in detail by Ambard and Trautmann (4) and Ferry (23).

deMan (18) made a comparison between the composition of milk serum as obtained by ultrafiltration, rennet whey preparation, and dialysis and confirmed that from 3.2% to 4.6% of the soluble material in milk is retained by the ultrafiltration membrane and this consisted mainly of calcium and citric acid.

The various ionic forms in which the soluble salts of milk are present can be calculated approximately from the analytical composition of milk serum and the dissociation constants of phosphorus, citric and carbonic acids, after allowance has been made for binding of calcium and magnesium to citrate as anionic complexes and to phosphate as undissociated salts. Dissociation constants for the citrate combinations are given by Hastings et al. (27) and for phosphate by Greenwald et al. (25) and Tabor and

Hastings (63). Calculations by Smeets (58) based on these constants suggest that about 35% of soluble calcium and magnesium are present as ions, about 55% bound to citrate and about 10% bound to phosphate in normal milk.

Although the concentration of the various ionic species can be roughly calculated from the general principles of equilibrium, there are very few which can be determined experimentally. Fortunately methods of measuring the concentration of calcium and magnesium ions considered to be the most important as regards influence on protein coagulation and milk processing in general, have been developed in recent years. Two methods in particular have found extensive use, the colorimetric murexide method of Smeets (58) and the ion exchange equilibrium method of Christianson et al. (15) and Van Kreveld and Van Minnen (65). The better known and more commonly employed Smeets' method depends on a progressive shift in the absorption maximum of murexide from 520 to 480 millimicrons as the calcium concentration is increased. Tessier and Rose (64) have improved the accuracy of the method and rendered it relatively independent of the exact murexide concentration employed by measuring the difference in the extinctions at 520 and 480 millimicrons instead of the extinction at 480 millimicrons alone. According to these authors the reproducibility of this modification is about ± 0.05 mmole of Ca^{++}/l for normal milk. Results are appreciably affected by ionic strength and by the concentration of sodium and potassium ions present, as Tessier and Rose demonstrated.

Smeets (58) found the calcium ion concentration of milk diffusate to be 2.7 ± 0.39 mmoles/1; Tessier and Rose (64) the very similar values of 2.5 - 3.4 mmoles/1.

Among other methods which have been proposed for the determination of calcium and similar cationic concentrations in milk is that of Affsprung and Gehrke (2) based on the use of ion exchange membrane electrodes. They reported the rather high value of about 5 mmoles/1 for the calcium concentration in milk. A further method developed in the same laboratory and known as the Resin Contact Time method, Baker, Gehrke and Affsprung (6), is reported to have furnished an average value of 23.8 mg/100 ml (5.95 mmoles/1) for ionic calcium (2), again distinctly higher than that obtained by the Smeets method (58).

The Alcohol Stability of Milk

The Alcohol Test:

The Alcohol test has been used in the past and is being used to some extent as a test for grading milk. The test is usually made by adding 2 ml of milk to 2 ml of ethanol in a test tube. The test tube is then closed with the thumb, inverted several times and examined. If no precipitate is formed the test is said to be negative and if a precipitate is formed the test is said to be positive. The strength of the alcohol used is usually 68 or 70 per cent by volume.

When the test first came into use in 1890, it was regarded largely as a measure of acidity. However, it was soon found that fresh milk from individual cows frequently coagulates and the test was dependent upon factors other than acidity. Nevertheless, the test was still used as it was thought that besides acidity, it detected other undesirable characteristics, such as colostrum milk, diseased udders, diseased cows and sensitivity to heat coagulation, etc.

The strength of alcohol necessary to cause coagulation can vary widely with different milks (41). The clots consist mainly of partially denatured casein still associated with calcium and calcium phosphate (12). Since the caseinate dispersion in milk is believed to be colloidally stable by virtue of the hydration and negative charge of the micelles (12), the coagulating effect of alcohol can be attributed to an initial dehydration followed by an aggregation of the stabilized micelles in the presence of calcium and magnesium ions, the bivalency of these cations confers on them a much greater coagulating power towards a negatively charged colloid than the other cations in the milk (39). The lowering of the dielectric constant of the medium by alcohol also helps in the aggregation of the micelles.

Sommer and Binney (61) noted that the addition of dipotassium phosphate or sodium citrate to milk, previously made unstable to ethanol by addition of calcium acetate or magnesium chloride, counteracted the destabilizing influence of the cations. These

results were considered to show the influences of salt balance on the stability of the caseinate complex to ethanol. No relation was found between the titratable acidity of fresh milk and its stability to ethanol, though developed acidity reduced stability. They observed also that when calcium carbonate was fed to three cows their milk became unstable to ethanol, even though the total calcium content of the milk was unaltered. They made a comparison of the effect of the salts on the alcohol coagulation shown by their study, and the effect of the salt on the heat coagulation as found by Sommer and Hart (60) and concluded that both the alcohol and heat coagulation are produced by an excess of calcium and magnesium over citrates and phosphates.

Benton and Albery (7) recorded an instance where the fresh bulk milk from a herd of fifty-four cows gave a precipitate with alcohol, presumably 70% alcohol, although the exact concentration is not clear from the paper; the milk of the fifty-four cows gave a positive reaction. The milk was apparently normal in every respect and the reaction could not be ascribed to feeding, stage of lactation, nor to any pathological conditions of the udders.

The most comprehensive study of the coagulation of milk by ethanol is that of Mitamura (41). He found that the mean alcohol strength required to coagulate milk was 80 per cent. In early lactation milk was very unstable to ethanol, but thereafter it gradually became more stable and remained at a fairly constant level of stability that was specific for each cow. Towards the end of

lactation, the milk from some cows became more stable to ethanol, but a decrease in stability was more common. Relatively unstable milks contained more soluble calcium, soluble magnesium and chloride, but less soluble inorganic phosphorus than those more stable to ethanol. Confirmation of the importance of bivalent ions was obtained by adding neutral salts to milk when it was found that the strength of ethanol that caused coagulation decreased as the amount of added bivalent cations increased. Neither pH nor the concentration of fat, colloidal calcium, colloidal phosphorus was related to stability to ethanol. Eilers (22) confirmed that milk was made less stable to ethanol by addition of calcium salts or acids and was made more stable by addition of bases or salts whose anions could form weakly dissociated calcium compounds.

Seekles and Smeets (56) showed that the 'Utrecht abnormality' of milk, i.e. an instability of the protein to heat and ethanol, was not related to total calcium content and suggested that a high concentration of calcium ions was the cause. This view was supported by the fact that when the concentrations of calcium ions in milk was reduced by adding alkali, or anions which could combine with the calcium, the stability of the milk to ethanol increased. Seekes and Smeets (56) claimed also that the 'Utrecht abnormality' could be successfully counteracted by supplementary feeding or injection of sodium citrate.

Further evidence that diet could influence the stability of milk to ethanol was provided by Echenique and Suarez (20, 21) who reported

that when cows ate plants rich in calcium, their milk was unstable to ethanol though normal in acidity. Also Hughes and Ellison (34) quoted a report that cows grazing on land with a high calcium content often secreted milk that was unstable to ethanol. Rowlands et al. (51) stated that only one of 618 fresh bulk milks coagulated with 60% ethanol, but with stronger solutions of ethanol the incidence of unstable samples rose sharply especially in limestone districts. Weimar (70) induced alcohol instability by feeding mouldy silage and ascribed it to physiological disturbances leading to chemical changes in milk.

Mitamura (41) was also able to induce temporary instability by feeding a ration composed entirely of viscid fermented soya bean cakes. Feeding protein rich or protein deficient rations for short periods or sudden changes in cows' fodder did not have any effect on the alcohol stability of the milk.

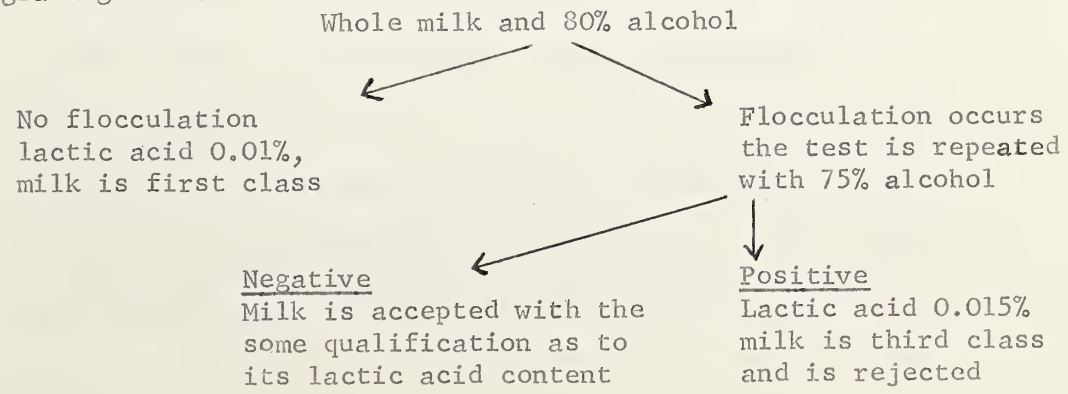
The work of Seekles and Smeets (57), Boogaerdt (11) and Smeets (58) supports the hypothesis that the concentration of bivalent cations is the most important factor governing the stability to ethanol of the caseinate complex in milk. However, in no investigation has a series of milks differing widely in stability to ethanol been analyzed in sufficient detail to enable a relation between stability and chemical composition to be definitely established.

Davies and White (16) made a detailed study of factors responsible for the alcohol instability and reviewed the literature

on the subject. They confirmed the findings of early workers (41). They found that samples of herd bulk milk were similar in stability to ethanol; the range of aqueous ethanol solutions required to coagulate the caseinate complex in an equal volume of milk was only 80-84%. Samples from individual cows showed a wide variation in stability; coagulation was caused by ethanol solutions ranging in strength from 66-90%. Colostrum was very unstable to ethanol, but stability rapidly increased during the post colostrum period to higher levels in mid-lactation. Late lactation and sub-clinical mastitis milk showed no definite bias to stability or instability. They also observed that the strength of ethanol required to coagulate the caseinate complex was inversely related to the concentration of ionized calcium in the milk.

The relationship between the concentrations of other milk constituents and stability to ethanol could be attributed to the interrelations of the concentrations of these constituents and the concentration of ionized calcium.

Heintzberger (28) made an attempt to relate acidity of the milk to the alcohol test and suggested the following test for grading milk:



He claimed that milk which had developed an appreciable amount of lactic acid, but had been neutralized to a normal degree of acidity and repasteurized, nevertheless showed a positive alcohol test. This form of adulteration could easily be detected. Results of the studies of Iizuka (33) on alcohol test positive milk with normal acidity showed that the average ionized calcium concentration of 114 out of 126 alcohol positive samples was 15.6 mg/100 ml which was 1.6 times that of normal milk.

Anderson (5) has suggested a simple and practical test for sour milk, which uses .05% solution of alizarin (dye) in 68% alcohol. When mixed in equal amounts with milk this solution develops a brown colour which changes to yellow as the acidity goes up. The main reason for the choice of this solution was that it not only colours the milk but also coagulates it at a higher acidity. This coagulation is very easily detected in the coloured medium. He states that the test could be used at the farm pick-up tankers and also with equal advantage as a platform test.

Rennet Coagulation of Milk

It has been known for many years that the coagulation of milk by rennet occurs in two stages. Recent investigations have shown that the essential reaction in the first or enzymic stage is the rapid liberation of non-protein nitrogen from α-caseinate with the formation of α-paracaseinate (3, 29, 42, 43). The temperature coefficient (Q_{10}) of this reaction at a pH typical of milk, 6.7, was

found to be about 1.9 between 1 and 30°C, and at constant rennet concentration and constant temperature the rate of the reaction increased as pH decreased from 6.7 to 5.5 (44). According to Pyne (47), the rate of the first stage of coagulation increases as the concentration of ionized calcium increases. It has been reported also that the hydration and charge of the caseinate micelles are reduced during the first stage (26).

In the second or non-enzymic stage, the α-paracaseinate, together with the unchanged β- and γ-caseinates coagulate provided calcium ions or other alkaline earth ions are present and the temperature is within the range 15 to about 45°C (70). The temperature coefficient of the second stage of coagulation is larger, namely 1.3 - 1.6 per °C (8). The magnitude of this temperature coefficient led Berridge (8) to suggest that the second-stage reaction is a heat denaturation of the paracaseinate micelles followed by their aggregation, with calcium ions acting as 'bridges'. Normally, all the α-caseinate is converted to α-paracaseinate before coagulation occurs (3, 42) but when the concentration of calcium ions is sufficiently large, a premature precipitation of incompletely formed paracaseinate can take place (48). The rate of the second stage of coagulation is appreciably increased by only a small increase in the concentration of calcium ions (46, 48). Pyne (48) states that pH has little influence on the second stage but according to Smith and Bradley (59) a decrease in pH accelerates the second stage as well as the first.

Much of the above information on the coagulation of milk by rennet was obtained by studying each stage separately. Of more practical interest are the attempts that have been made to relate the over-all time taken by both the enzymic and non-enzymic reactions to form a coagulum, i.e. the renneting time of milk, to the chemical composition of milk.

No close or consistent relation has been established between renneting time and the concentration of total or soluble calcium in milk. There is evidence that milks with long renneting times tend to be low in calcium content (14, 24, 30, 43), but Ling (35) found that the renneting time of milk increased as the soluble calcium content increased, and he suggested that salt-balance might influence renneting time. On the other hand, it is well known that when calcium salts are added to milk, renneting time decreases (8, 13, 40, 59, 67, 68), and that when some calcium is inactivated or removed, renneting time increases (53, 68). When about 20% of the calcium is removed, coagulation does not occur (53). White and Davies (71) also found that milk containing a relatively high concentration of ionized calcium coagulated quickly with rennet.

The addition of magnesium ions is also known to decrease renneting time (68). Berridge (9) states that the accelerated coagulation in the presence of added bivalent cations is due partly to a decrease in the pH of milk, but with calcium ions it is known that the accelerating effect persists even when the pH is kept

constant. There is the possibility that renneting time may depend to some extent on the concentration of trace metals in milk since the addition of small amounts of some heavy metal salts (e.g. Mn, Cd, Zn) shortens renneting time and the addition of others (e.g. Co, Cu, Ni) lengthens it (39).

It is well known that the acidity of milk is an important factor governing renneting time, although there appears to be a lack of direct experimental evidence showing how the natural variation in the acidity of milk from individual cows is related to the variation in renneting time. Some evidence of a tendency for renneting time to increase as acidity decreases has been provided by Holm et al. (30); Ling (35, 36) and Sanders et al. (52) have given average values for groups of milks from individual cows showing that there was a progressive increase in renneting time as pH increased from 6.32 to 6.80. The longest average renneting time was approximately nine times greater than the shortest. There is ample indirect evidence of an inverse relation between acidity and renneting time, e.g. the addition of acid to milk or the natural souring of milk shortens renneting time (36, 40, 68).

Presumptive evidence of an inverse relation between acidity and renneting time is the increase in renneting time with advancing lactation (pH increasing) (40, 71) and the long renneting times common with sub-clinical mastitis milk (pH often high) (19, 52, 62). White and Davies (71) from their work on rennet coagulation of milk, found that the property of milk most closely related to renneting time was

constant. There is the possibility that renneting time may depend to some extent on the concentration of trace metals in milk since the addition of small amounts of some heavy metal salts (e.g. Mn, Cd, Zn) shortens renneting time and the addition of others (e.g. Co, Cu, Ni) lengthens it (38).

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acidity. As pH increases from 6.4 to 7.2 there was a curvilinear increase in renneting time from 1.5 to 13 minutes. Titratable acidity was related to renneting time in the opposite way to pH. Sebela and Pavel (55) confirmed that the relation between the native acidity and renneting time is a negative one.

There are conflicting views on whether differences in the caseinate complex are partially responsible for the variations in the renneting time of milks. The possibility of an inverse relation between renneting time and the particle size of the caseinate complex is also suggested by reports that when milk is homogenized the size of the caseinate micelles increases (31), and renneting time decreases (31, 72). Chevalier et al. (14) and Mocquot et al. (43) believe that the physical and chemical constitution of the caseinate complex as well as the aqueous phase determine the renneting time of milk. Pyne (47), from his earlier work, believed that the composition of the aqueous phase, and not that of the caseinate complex determines renneting time. But in their recent paper, Pyne and McGann (50) stated that the sensitivity of the rennet-altered calcium caseinate, calcium phosphate complex of milk to calcium ions is directly related to the colloidal calcium phosphate content of the complex, and the duration of the second stage of the rennet coagulation is accordingly related inversely thereto.

Schipper and Mulder (54), in their most recent work on the composition of the caseinate phosphate complex, found that calcium

caseinate dispersions became unstable when complex calcium phosphate (Ca:P = ca. 1.4) is precipitated on the casein. They observed the formation of microscopically visible particles of calcium caseinate phosphate complex resembling the casein particles of milk and giving the impression of being a coacervate. When much phosphate is precipitated on it, the casein dispersion coagulates. They believe that the stability of a dispersion is not only determined by the continuous phase, but that the dispersed phase has to be considered also. According to these authors, theories taking into account the concentration of ionized calcium only are one sided and are not yet proved quantitatively.

Much information is now available on the factors which regulate each stage of coagulation and also on the effect of added substances on the renneting time of milk, but the relation between the chemical composition of the milk and renneting time has not been adequately examined. The lack, until recently, of a suitable method for estimating calcium ions has made it impossible to determine the influence of the concentration of this milk constituent on renneting time and only average data are presented in most extensive comparison of pH and renneting time so far reported (52, 71).

EXPERIMENTAL METHODS

Description of Milk Samples

One hundred and seven samples of individual milks were obtained from the University of Alberta dairy herd of pedigree Holstein cows. The samples were taken from fifteen individual cows in different seasons of the year. All of the samples from individual cows were taken from the complete evening milking.

Collection of Samples

Samples from individual cows were collected by connecting a sampling bottle between the milk meter and the pipeline. The arrangement is shown in Fig.1.

The milk from the milking machine goes to the milk meter, which clicks after every quarter pound of milk passing through it to the pipeline. On its way to the pipeline, the milk passes through the aluminum pipe which fits horizontally into an opening in the rubber stopper on top of the sampling bottle. There is an opening at the bottom of the rubber stopper which meets a hole drilled half way into the aluminum pipe and a capillary tube of about two inches in length is pushed into this opening in the stopper to meet the opening in the aluminum pipe. Some of the milk, while passing through the aluminum pipe drips into the bottle through the capillary tube. This goes on until the

Fig.1. Sampling device for collection of milk samples from a pipeline system during milking.

milking of the cow is finished. Thus, a small fraction of milk going to the pipeline is collected into the bottle each time the milk meter clicks and the sample is representative of the whole milking. The volume of each sample taken was about 500 ml.

Analysis of Samples

The samples collected in the evening were stored at 4°C over night and the analysis of the samples was started the next morning.

Protein Stability Test

1. Ethanol: The stability of milk protein to ethanol was determined by finding the strength of ethanol solution which, when added to an equal volume of milk, caused the formation of clots. Thirteen aqueous solutions of ethanol were used covering the range 66 - 90% (v/v) ethanol in 2% intervals. The procedure was as follows: 2 ml of milk were pipetted into a test tube, 2 ml of 90% ethanol added and the tube was closed with the thumb and inverted four or five times. The mixture was poured into a glass petri dish and examined for the presence of clots. The test was repeated with the other ethanol solutions, in order of decreasing strength until coagulation did not occur. The strength of the weakest ethanol solution that caused the formation of clots was recorded. The above test was carried out at room temperature.

2. Rennet: The stability of milk protein to rennet was determined by the time required to coagulate the milk upon addition of an equal volume of the enzyme solution. The clotting time was measured by an automatic clot-timer. The instrument used was a Mechrolab Model 201 Clot-timer* and is shown in Fig.2.

The samples and the enzyme solution are forewarmed to 37°C in the wells of the thermostatically controlled block. 0.2 ml of the sample is introduced into the sample cup and an equal volume of rennet solution is added. At the moment of the addition of rennet the timer is started.

The rotor has a dual function: it stirs the reaction mixture at a constant speed of 60 r.p.m. and as soon as the mixture coagulates the rotor picks up a clot from the mixture which is deposited on a set of two electrodes. This closes the detector circuit and stops the digital timer. The clotting time is then read on the timer dial and recorded.

The rennet solution used was prepared from powdered rennet. One gram of powdered rennet was made up to 100 ml with 5% sodium chloride solution.

The coagulation time of each sample was determined in duplicate, and the mean calculated.

* Manufactured by Mechrolab Inc., Mountain View, California, U.S.A.

Fig.2. Automatic clot-timer used for the determination of rennet clotting time of milk.

Chemical Analysis of the Samples

The milk samples were analyzed for the following salts in their various fractions -

Calcium:	Total Calcium
	Soluble Calcium
	Ionic Calcium
Magnesium:	Total Magnesium
	Soluble Magnesium
Phosphorus:	Total Acid Soluble Phosphorus, i.e. colloidal inorganic phosphorus + soluble inorganic phosphorus.
	Soluble Inorganic Phosphorus
	Colloidal Inorganic Phosphorus
Citric Acid:	Total Citric Acid
	Soluble Citric Acid

To partition the calcium, magnesium, phosphorus and citric acid, it was necessary to prepare an ultrafiltrate (or diffusate or rennet whey) and trichloroacetic acid (TCA) filtrate from each sample of milk. The ultrafiltrate was obtained by filtering milk through cellophane in an ultrafilter similar to the one designed by Ambard and Trautman (4). The ultrafilter is shown in Figs.3 and 4. It consists of two stainless steel halves. The bottom half has fourteen bolts welded on its margin which pass through

Fig.3. Ultrafilter used for separation of the soluble fraction of milk salts.

Fig.4. Ultrafilter disassembled.

the holes on the top half and thus the two can be clamped together with the help of wingnuts. A metal plate, which has a screen on one side and is perforated on the other sits on the bottom half and a cellophane disc is placed on this screen. The top is then clamped on.

The milk is poured through the side inlet in the top half of the ultrafilter, and the inlet is closed. The top of the ultrafilter is connected to the air line. One hundred ml of milk sample was used for ultrafiltration under a pressure of twenty-five pounds per square inch. About 15 ml of ultrafiltrate was collected in seventy minutes and used for analysis.

The TCA filtrate was prepared as follows: 20 ml of milk was mixed with an equal volume of 20% trichloracetic acid. The mixture was shaken vigorously for a few seconds and was allowed to stand for ten minutes and filtered through Watman No.1 paper. The filtrate was used for analysis.

Determination of Calcium

Total Calcium: The total calcium was determined in TCA filtrate by the improved complexometric method of Abd-el-Raheem (1) with the following modifications:

1. Deionized water was used throughout.

2. .01 N ethylenediaminetetracetic acid (EDTA) was prepared from B.D.H. concentrated volumetric solutions.

3. Indicator used was Patton and Reeder's reagent, 2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphtylazo)-3-naphtoic acid. One gram of the above reagent was finally ground with 100 grams of sodium chloride.

Soluble Calcium: The amount of soluble calcium was determined by titrating one ml of the ultrafiltrate with EDTA in the same manner as the TCA filtrate.

Ionic Calcium: The method described by Walser (69) for the determination of free calcium ions in the body fluids was modified and used for the determination of calcium ions in milk ultrafiltrate.

Method

Reagents:

Deionized water was used throughout.

Tris Buffer: A buffer solution pH 6.8 containing .16 M Tris (Hydroxymethyl) amino methane and .16 M sodium chloride was made by dissolving 19.382 gm of Tris and 9.352 gm of sodium chloride in about 900 ml of water. The pH was brought to 6.8 by adding hydrochloric acid, and the volume made up to 1000 ml.

Calcium Stock Solution: Was prepared by dissolving pure dry calcium carbonate in a

slight excess of hydrochloric acid. The excess of the acid was then neutralized by potassium hydroxide to bring the pH to 6.8. The stock solution contained 5 mg Ca^{++}/ml.

Calcium standards containing 10, 20, 30 and 40 mg Ca^{++}/100 ml were prepared from the stock solution by diluting it 1 to 50, 2 to 50, 3 to 50 and 4 to 50 with .16 M sodium chloride.

Murexide: (Acid ammonium purpurate) 0.03% freshly prepared, dissolved in Tris buffer pH 6.8 and 0.005 M.

Procedure:

Two ml of the ultrafiltrate was transferred to a colorimeter tube, then 2 ml of .16 M sodium chloride and 2 ml of Tris prepared as above was added, and absorbance determined in a Bausch and Lomb Spectronic 20 spectrophotometer. Then .2 ml of the above dye solution was added and mixed and absorbance determined again.

Standard solutions read at the same time were prepared as follows: Two ml of each calcium standard and 2 ml of .16 M sodium chloride were mixed with 2 ml of Tris buffer. Then, .2 ml of dye solution added. The blank contained 4 ml of

.16 M sodium chloride and 2 ml of Tris buffer and gave the same reading as distilled water.

The absorbance of the ultrafiltrate Tris mixture without the dye was subtracted from the absorbance after the addition of the dye. In addition, the absorbance of the dye containing blank was subtracted. The resulting value represents the change in absorbance (A-Ao) due to the calcium originally present in ultrafiltrates or standards.

The standard curve obtained by plotting the change in absorbance (A-Ao) and calcium ion concentration is shown in Fig.5. The standard curve was run with each group of unknown samples.

Determination of Magnesium

TCA filtrate and ultrafiltrate were used for the determination of total and soluble magnesium respectively. The amount of magnesium was determined by difference using Eriochrome black T as the indicator.

Determination of Inorganic Phosphorus

Total inorganic phosphorus was determined in TCA filtrate and soluble inorganic phosphorus in ultrafiltrate by the method of Polley (45).

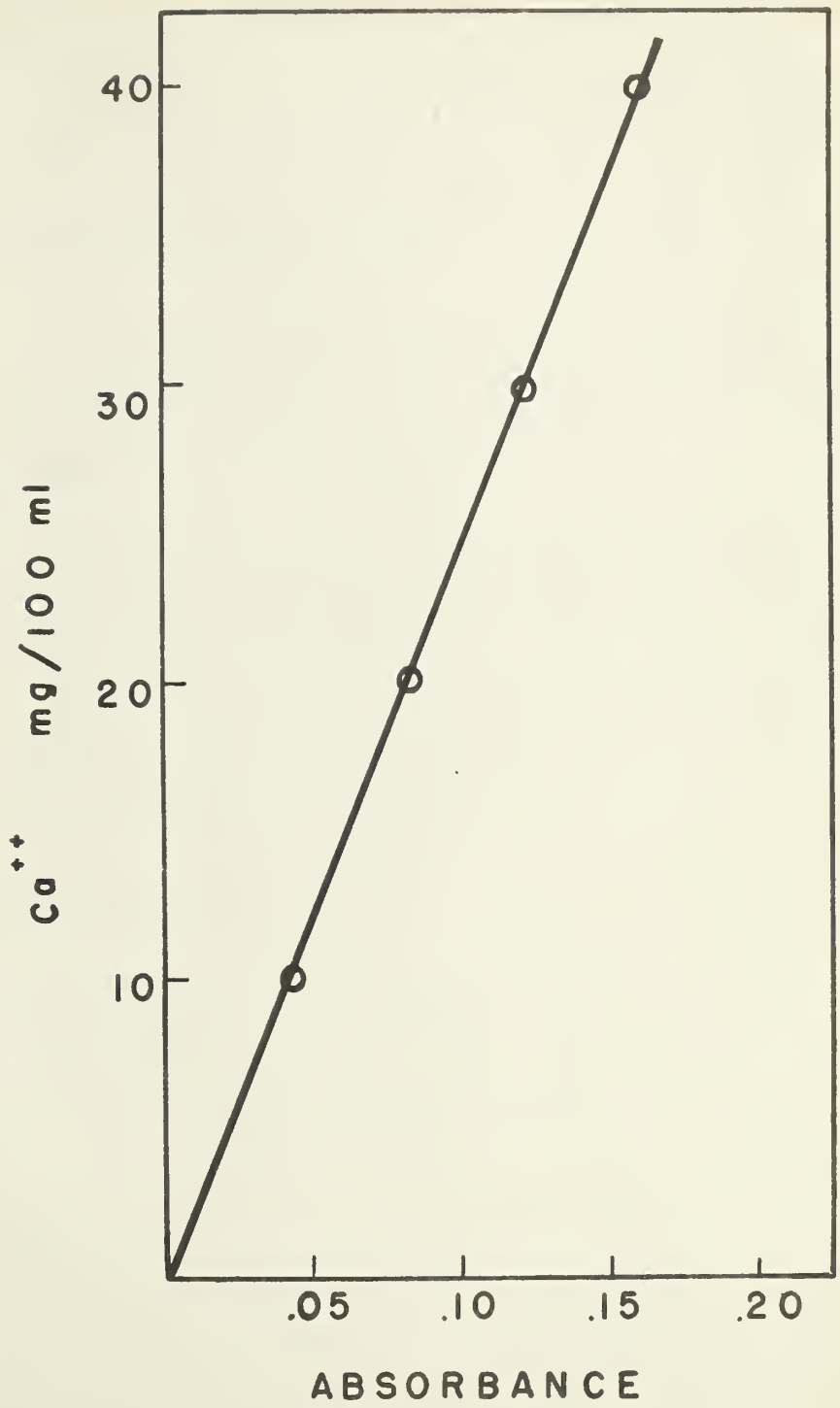

Fig.5. Standard curve for the determination of the concentration of ionic calcium in milk.

The TCA filtrate and the ultrafiltrate samples were diluted to have the phosphorus content between .2 to 1.4 mg/ 100 ml.

The calibration curve using mono potassium phosphate standards is shown in Fig.6.

Determination of Citric Acid

Total citric and soluble citric acid were determined in TCA filtrate and ultrafiltrate respectively by the improved pyridine acetic anhydride method (37).

The TCA filtrate and ultrafiltrate were diluted to have a citric acid content between 50 and 250 µg/ml.

The calibration curve using anhydrous citric acid (monohydrate) is shown in Fig.7.

Statistical Analysis

Much of the statistical analysis (means, standard deviations, correlations, least squares) was done at the University Computing Centre using punch cards and I.B.M. 1620 digital computer.

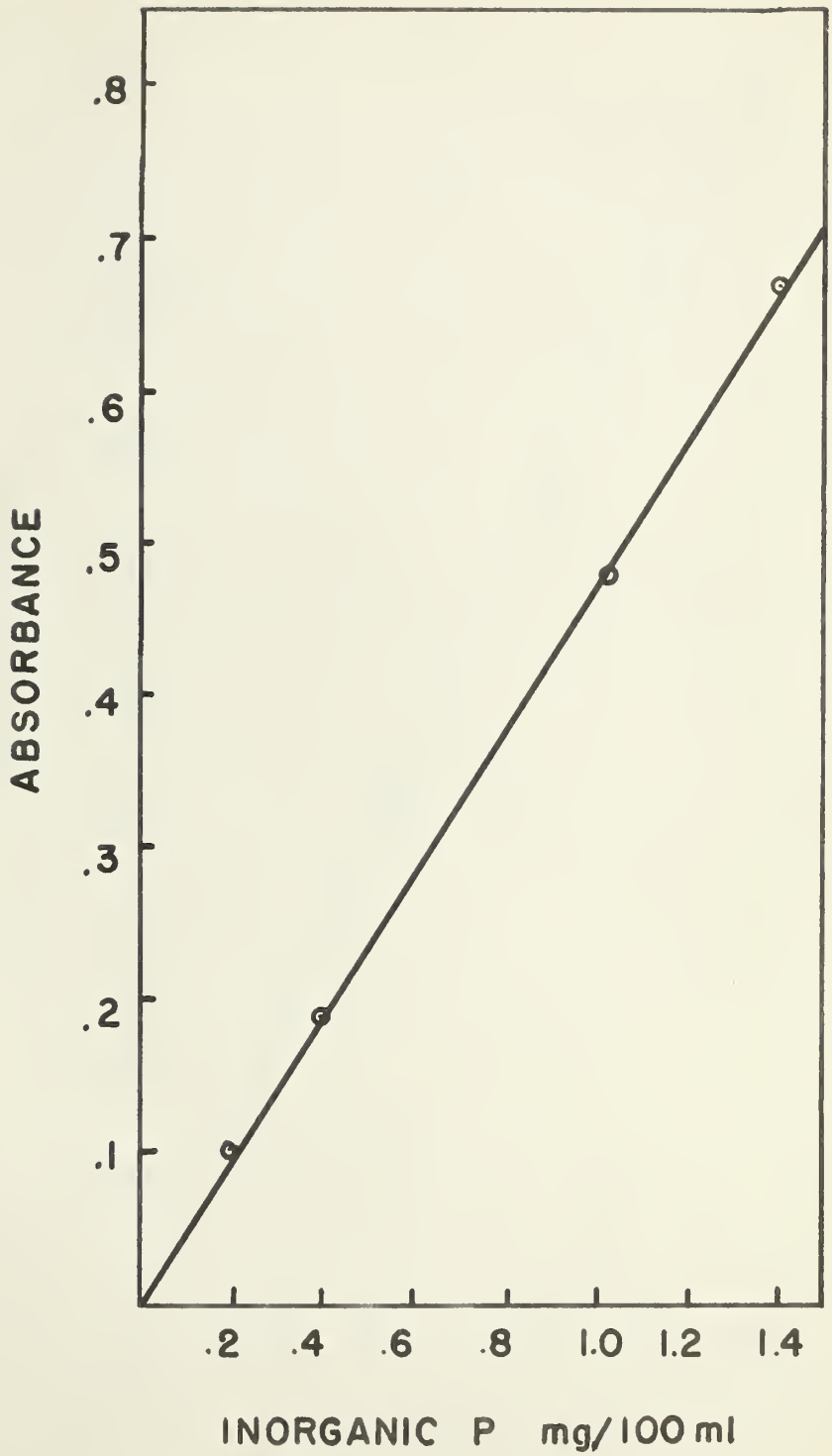

Fig.6. Standard curve for the determination of inorganic phosphorus in milk.

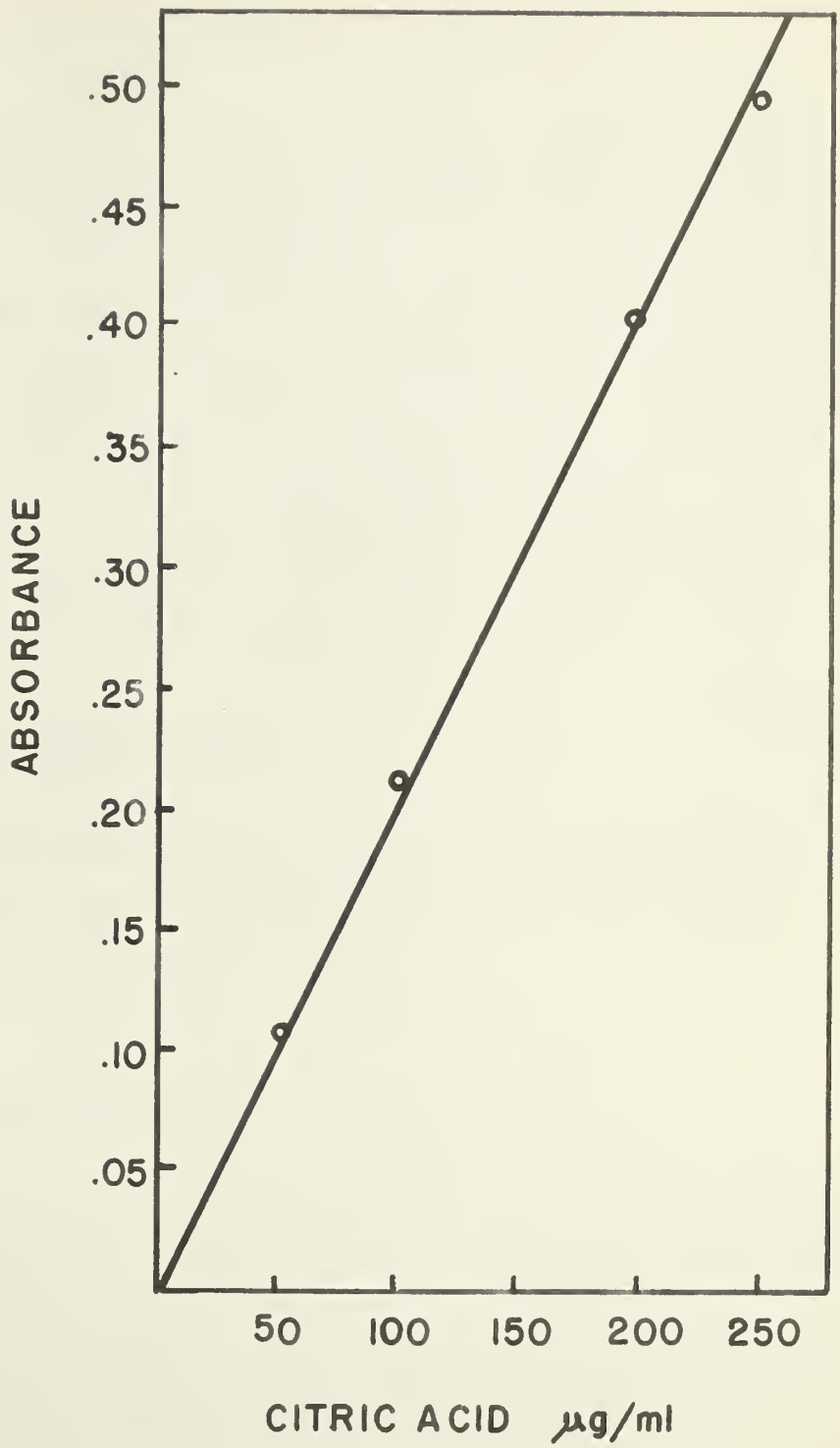

Fig.7. Standard curve for the determination of citric acid in milk.

RESULTS

Range of Stability to Ethanol

Table 1 shows that varying degrees of stability were found in the milks from individual cows. Some samples were coagulated by 66% ethanol while others required up to 88% ethanol for coagulation. The overall average for the group was 69.3% ethanol. As will be seen from Table 1, 44 samples coagulated with 66% ethanol. The rapidity and completeness suggested that they would have coagulated with an even weaker solution of ethanol. For calculation purposes the percentage of ethanol was taken as 64 for these samples, as is usual with the alcohol test.

Range of Renneting Times

A wide variation was found in the renneting times of the milks examined. The range classification is shown in Table 2. The mean of 107 samples was 87.93.

Chemical Composition

The range classification, mean and standard deviation for total soluble and ionic calcium is shown in Tables 3, 4 and 5, respectively.

Tables 6 and 7 show the range classification, mean and standard deviations for total and soluble magnesium.

Tables 8 and 9 show the amounts for total and soluble citrate.

The range classification,mean and standard deviation for total inorganic, soluble inorganic and colloidal inorganic phosphorus are given in Tables 10, 11 and 12, respectively.

It will be seen from Tables 1 to 12 that there is a wide variation in the chemical composition for various salts determined in the samples.

In order to study the relation between the stability and various milk constituents and between constituents themselves, the correlation coefficient between various variables, taking two at a time (X and Y shown in Table 13) were determined.

The level of significance was also calculated from the corresponding 't' values.

From Table 13, there appears to be a relationship between the various milk constituents and stability, and the relationship is negative between (a) ionic calcium and alcohol %, (b) ionic calcium and clotting time, (c) (ionic calcium x colloidal inorganic P) and alcohol %, and (d) (ionic calcium x colloidal inorganic P) and clotting time, which would be expected, but the relationship is by no means a linear one.

The correlation between the salt balance and alcohol stability is negligible which goes to contradict the salt balance theory of Sommer & Binney (61).

The least square line was plotted between the following parameters -

Total calcium	vs.	Soluble calcium
Soluble calcium	vs.	Ionic calcium
Alcohol %	vs.	Clotting time

They are shown in Figs.8, 9 and 10.

In order to compare the stability of milk samples before and after the cows went to pasture, 24 samples from 8 cows were taken in the months of March and April (i.e. off pasture) and also in June and July (i.e. on pasture). The mean of various determinations on 24 samples off and on pasture is shown in Table 14.

It will be seen that the mean for alcohol stability and clotting time is slightly higher on pasture but the difference is not significant. The corresponding 't' values being 0.397 for alcohol stability and 0.198 for the clotting time.

There is some variation in chemical composition particularly in the amounts of ionic calcium, citrate and phosphate but it cannot definitely be attributed to change in feeding as other factors as stage of lactation and season of the year are also operating.

Table 1. Alcohol stability of 107 samples with range classification, mean and standard deviation.

Alcohol (%) range	Number of Samples
66 or less	51
67 to 70	15
71 to 74	22
75 to 78	12
79 to 82	4
83 to 86	13
Mean	69.3
Standard deviation	5.84

Table 2. Clotting time of 107 samples with range classification, mean and standard deviation.

Clotting time range (seconds)	Number of Samples
60 - 69	8
70 - 79	32
80 - 89	33
90 - 99	16
100 - 109	12
110 - 119	1
120 - 129	4
130 - 139	1
Mean	87.9
Standard deviation	13.7

Table 3. Total calcium content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
90 - 99	5
100 - 109	21
110 - 119	23
120 - 129	36
130 - 139	12
140 - 149	10
Mean	119.9
Standard deviation	12.8

Table 4. Soluble calcium content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
25 - 29	5
30 - 34	21
35 - 39	36
40 - 44	38
45 - 49	6
50 - 54	1
Mean	38.0
Standard deviation	4.6

Table 5. Ionic calcium (Ca^{++}) in 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
5 - 9.9	1
10 - 14.9	1
15 - 19.9	23
20 - 24.9	59
25 - 29.9	22
30 - 34.9	1
Mean	22.0
Standard deviation	3.4

Table 6. Total magnesium content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
6 - 7.9	3
8 - 9.9	4
10 - 11.9	21
12 - 13.9	54
14 - 15.9	16
16 - 17.9	8
18 - 22.9	1
Mean	12.7
Standard deviation	2.2

Table 7. Soluble magnesium content in 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
6 - 6.9	5
7 - 7.9	10
8 - 8.9	35
9 - 9.9	19
10 - 10.9	23
11 - 11.9	3
12 - 12.9	8
13 - 13.9	4
Mean	9.4
Standard deviation	1.6

Table 8. Total citric acid content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
100 - 119	3
120 - 139	9
140 - 159	15
160 - 179	37
180 - 199	31
200 - 219	9
220 - 239	2
240 - 264	1
Mean	172.7
Standard deviation	25.7

Table 9. Soluble citric acid content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
80 - 99	2
100 - 119	9
120 - 139	19
140 - 159	57
160 - 179	24
180 - 199	13
200 - 219	3
Mean	153.3
Standard deviation	24.6

Table 10. Total inorganic P content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
40 - 49.9	3
50 - 59.9	21
60 - 69.9	58
70 - 79.9	22
80 - 89.9	3
Mean	64.9
Standard deviation	7.7

Table 11. Soluble inorganic P content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
20 - 24.9	3
25 - 29.9	26
30 - 34.9	39
35 - 39.9	30
40 - 44.9	9
Mean	33.0
Standard deviation	4.7

Table 12. Colloidal inorganic P content of 107 samples with range classification, mean and standard deviation.

Range (mg/100 ml)	Number of Samples
10 - 14.9	1
15 - 19.9	3
20 - 24.9	11
25 - 29.9	24
30 - 34.9	31
35 - 39.9	35
40 - 44.9	8
45 - 49.9	4
Mean	32.0
Standard deviation	6.7

Table 13. The relationship between the various variables giving correlation coefficient, 't' value and level of significance.

X	Y	Correlation coefficient 'r'	't' value	Significance level
Ionic Ca	Alcohol %	-0.349	3.815	1%
Ionic Ca	Clotting time (Sec.)	-0.265	2.810	1%
* Salt Balance	Alcohol %	-0.114	1.173	-
Salt Balance	Clotting time (Sec.)	-0.048	0.494	-
(Ionic Ca x colloidal inorganic P)	Alcohol %	-0.359	3.947	1%
(Ionic Ca x colloidal inorganic P)	Clotting time (Sec.)	-0.275	2.727	1%
Total Ca	Soluble Ca	0.429	4.870	1%
Total Ca	Ionic Ca	0.305	3.282	1%
Soluble Ca	Ionic Ca	0.615	7.989	1%
Alcohol %	Clotting time (Sec.)	0.487	5.714	1%

* Calculated as (citric acid + phosphorus) - (calcium + magnesium)

Fig.8. Relation between the total and soluble calcium content in milks from individual cows.

Fig.9. Relation between the soluble and ionic calcium content in milks from individual cows.

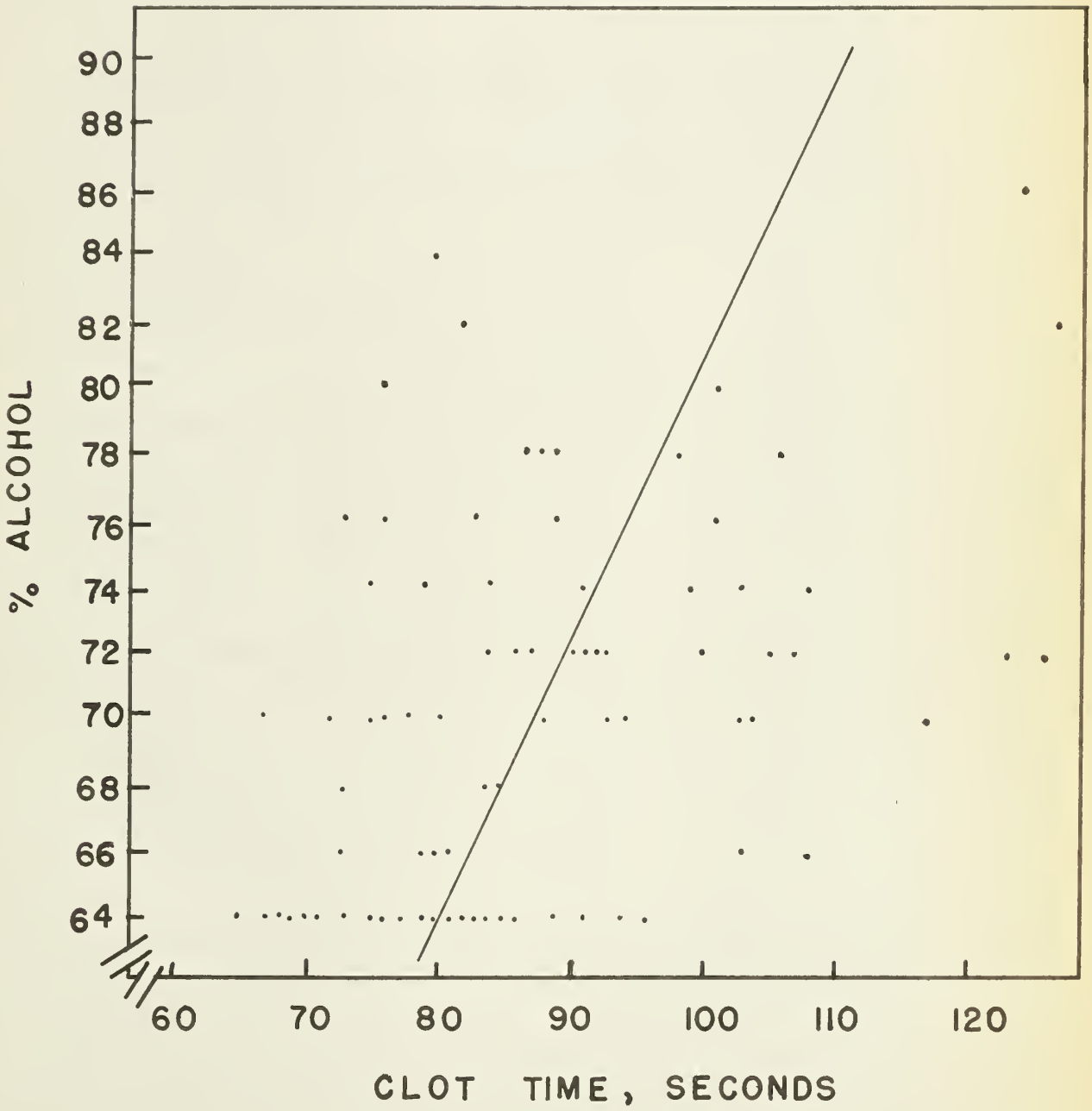

Fig.10. Relation between clotting time and the strength of ethanol required for coagulation in milks from individual cows.

Table 14. The mean values for various determinations for 24 samples off and on pasture.

Variable	Off pasture (mean)	On pasture (mean)
Alcohol %	72.3	74.3
Clotting time (Sec.)	86.6	89.2
Total Ca	113.2	112.2
Soluble Ca	35.4	34.8
Ionic Ca	22.4	18.8
Total Mg	12.75	12.75
Soluble Mg	9.58	8.77
Total citric acid	170.4	147.8
Soluble citric acid	151.2	129.5
Total inorganic P	65.7	60.4
Soluble inorganic P	37.0	32.5
Colloidal inorganic P	31.8	28.8

DISCUSSION

Stability to ethanol

The range of ethanol solutions required to coagulate the caseinate complex in 107 samples of milk from Holstein cows was very similar to that found by Mitamura (41) for 2704 milks from cows of several breeds, and Davies and White (16) for 132 samples from Ayrshire cows. The average strength of ethanol causing coagulation was much lower (69.3%) than found by these workers (16, 41), the average in their case being 80%. This could be due to many factors other than chemical composition such as breed, stage of lactation, seasonal effects, feeding and the environmental differences.

Relation of alcohol stability to chemical composition

There appeared to be no linear correlation between the concentration of ionic calcium and stability to alcohol as suggested by Davies and White (16). The correlation coefficient was found to be -0.349 which suggests that there is some negative relationship the nature of which could not be determined and this could be due to the fact that this is just one of the many factors affecting the alcohol stability of caseinate complex.

The salt balance theory of Sommer and Binney (61) did not prove effective in describing the stability of the caseinate complex to

ethanol. The salt balance theory was based on the fact that the addition of calcium and magnesium made the milk alcohol positive and citrates and phosphates counteracted this effect. Thus from their experiments Sommer and Binney concluded that there existed a balance between these four salts and their ratio in milk will determine its alcohol stability, but it is realized now that the addition of these salts in the milk will upset the original salt balance and in a complex system such as milk will induce other changes, e.g. acidity, which will have pronounced effect on the stability of the milk. Addition of these salts should not therefore be considered as a test for determining the influence of the same salt originally present in milk. In our experiments the salt balance showed no relationship to the alcohol stability which is contradictory to what would be expected according to the salt balance theory. There were other factors which showed some relationship (ionic Ca x colloidal inorganic phosphate) to ethanol stability and again this could just be one of the many factors.

Thus the general conclusion from the above could be summed up as follows.

There is no single factor which could determine the variation in ethanol stability of caseinate complex, but there are many factors which are possibly related to alcohol stability of the caseinate complex in one way or another, and the sum total of these would determine the resultant stability of the caseinate complex.

The small variations in stability and chemical composition of samples off and on pasture could be due to many factors. According to Jenness and Patton (34) there is no great variation in chemical composition of milk due to change in feeding, though there are contradictory reports (20, 21, 32, 41, 51, 70).

Rennet coagulation

All of the 107 samples of milk coagulated with rennet and the range of clotting times by using the method described earlier was not found as wide (60 - 135 secs.) as reported by White and Davies (1.4 - 12.9 min.) (71), in which they used the method of Berridge (10).

Relation between clotting time and alcohol stability

It has been known for some years that addition of certain ions such as calcium and magnesium increase while citrates and phosphates decrease both alcohol stability and clotting time and this would lead to the prediction that the two parameters may be interrelated.

Sanders et al. (52), from their observations on 300 samples of milk, reported that milks stable to rennet were usually stable to alcohol. Ling (35) from his work on the composition of milk and whey also suggested that conditions known to influence the alcohol coagulation of milk may also affect the rennet coagulation.

Sommer and Binney (1923) also reported that the prepared rennet caused a positive reaction in the alcohol test. In our findings there was a moderate degree of correlation between clotting time and ethanol stability of the samples examined, which is in general agreement with the above suggestions.

Clotting time and chemical composition

The coagulation of milk by rennet has long been believed to depend greatly on the composition of the salts of milk and in particular on the concentration of ionized calcium. In most of the reports supporting the above statement, observations were made on addition of calcium to the milk which decreased the clotting time (8, 13, 40, 59, 67, 68), or removal of calcium which increased the clotting time (53, 68). White and Davies (71), on the other hand, found no relation between the renneting time and the chemical composition of milk.

As mentioned earlier, additions of the salts to or their removal from milk will upset the original salt balance in milk and will consequently lead to other changes including the effect on the clotting time. Those reports (8, 13, 40, 53, 59, 67, 68) that do not take this into account, cannot be considered as very reliable.

A recent report by Pyne and McGann (50) suggests that the second stage of rennet coagulation is related not only to the amount of ionized calcium but also to the colloidal phosphate content of milk. The evidence was obtained by removing colloidal phosphate from the milk.

Schipper and Mulder (54) considered that theories taking into account the concentration of ionized calcium only are one sided.

Our findings support the view that there appears to be no correlation between one single constituent of milk and the clotting time and that clotting time follows a trend very similar to alcohol stability in that the various constituents of milk determined show some relationship to clotting time of the milk, but the relation between them is not a linear one.

Total calcium and soluble calcium

Total calcium in milk is the sum total of the calcium distributed in various fractions. At the normal pH of the milk, the milk is unable to dissolve all of the calcium present in it, and as a result a greater part of this mineral exists in the colloidal form. It would be logical, therefore, to assume that the distribution of calcium in various fractions will be proportional to the total amount present. A moderate degree of correlation, in fact, was found between the total calcium and soluble calcium contents of the milk.

Soluble calcium and ionic calcium

The soluble calcium in milk can be present in the form of complex ions or free ions. The amount of free calcium ions would be expected to increase or decrease proportionately to the

amount of soluble calcium present. In our experiment a marked degree of correlation was found between the soluble calcium and ionic calcium content of milk.

It is clear from the preceeding discussion that despite the detailed nature of the chemical analysis for salt composition in this investigation, no adequate explanation was found for the variation in coagulation times of the milk.

The inability to relate closely the chemical composition of the milks to their alcohol or rennet stability suggests that the stability of the milk is not related to one single factor in the chemical composition of milk, but a combination of the various variables determined can affect the stability to a large extent. It is not possible, therefore, to establish the relation between the chemical composition and the stability of the caseinate complex until it is precisely known in what way and to what extent the various factors in the chemical composition will affect one another; and their resultant effect will determine the stability of the caseinate complex in milk.

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